International Journal of Scientific Research and Engineering Development-– Volume 5 Issue 4, July-August 2022
Available at www.ijsred.com

RESEARCH ARTICLE OPEN ACCESS

Screening, Purification and Characterization of an Extremophile (Bacterial) Lipase from Tattapani, District Mandi, Himachal Pradesh, India

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Abstract

Himachal Pradesh itself is very rich in flora & fauna and is a hot spot of microbial biodiversity. To date, only a few research papers are available as immediate information about the production and purification of lipases from hot water-springs *Bacillus licheniformis*. Generally, lipase production is organism-specific and it is released during the late logarithmic or stationary phase. Purification of the enzyme is essential in industries such as fine chemicals, pharmaceuticals, and cosmetics, and also for understanding the 3-D structures and the structure-function relationships of proteins. The lipase purification was performed by ammonium sulphate precipitation (80%) dialysis and Octyl-sepharose column chromatography. The Octyl purified lipase was loaded in Native-PAGE. The molecular weight was determined as 38 kDa against the marker used.

Keywords —Lipase, Purification, Octyl-sepharose, Native-PAGE, Molecular weight

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I. INTRODUCTION

Himachal Pradesh itself has very vast flora fauna and is a hot spot of microbial biodiversity. There are number of places in the lap of Western-Himalaya where natural hot water springs exist. These hot water springs are goldmines for microbial biodiversity just like Thermus aquatic (Taq polymerase). However, in this study we will talk about the hydrolytic enzyme which emerged as one of the major biocatalysts with evident potential for contributing to the multi-billion-dollar under exploited lipid technology bio-industry and have been used in *insitu* lipid metabolism and *exsitu* multifaceted industrial applications[1].Purification of

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the enzyme is essential in industries highest pure form chemicals, pharmaceuticals/drugs & cosmetics and also for understanding the 3-D structures and the structure-function relationships of proteins[2]. Various purification strategies standardized and developed for lipase have been reviewed several times highlighting clearly the significance of designing optimal purification scheme for various microbial lipases[3,4]. For certain applications, such as synthetic reactions in pharmaceutical industry, further purification is needed. Since lipases are known to be hydrophobic in nature, having large hydrophobic surfaces around the active site, the purification of lipases may best be achieved by opting for affinity chromatography, such as hydrophobic interaction chromatography[5].

II. Materials and Methods

II.(A). Chemicals

$NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, $FeSO_4.7H_2O$, $(NH_4)_2SO_4$, Celite-545 (S.D. Fine–Chem. Ltd., Hyderabad, India); yeast extract and gum acacia (Hi-Media Laboratory, Ltd., Mumbai, India); methanol, ethanol, butanol, sucrose, KCl, KI, KNO_3, isopropanol, ammonium persulphate, 2-mercaptoethanol, HCl, bromophenol blue and molecular sieves (3Å X 1.5 mm; Merck-Ltd., Mumbai, India); p-nitrophenyl formate (p-NPF), p-nitrophenyl acetate (p-NPA), p-nitrophenyl benzoate (p-NPB) p-nitrophenyl caprylate (p-NPC), p-nitrophenyl laurate(p-NPL), p-nitrophenyl palmitate (p-NPP) from Alpha-aesar, Heysham, England. n-Hexane, Silica gel (0.040-0.063 mm, 230-400 mesh) acetic acid and Triton–X100, Tween-20, 40 and 80 (Qualigens Chemicals, Mumbai, India); phenyl methylsulphonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), sodioum lauryl sarcosine (SLS), acrylamide,bis-acryl-amide (N,N-methylene-bis-acrylamide) glycerol, glycine and Tris (2-hyroxymethyl-2-methyl-1,3-propanediol) from Sigma Chemicals Co., USA. All chemicals were of analytical grade and were used as received.

II.(B). Microorganism

For the present study, water samples were collected from hotwater-spring of Tattapani(Distt. Mandi, Himachal Pradesh, India) in sterile containers by authour. For the isolation of lipolytic microorganisms, 100 µl of water samples were plated on triolien and tributyrene agar plates. The formation of clear zone around the colony on the plate was considered as lipolytic microorganisms. In total, 101 isolates were found. Microorganisms which formed large clear zone around the colony were assayed for lipase activity (Winkler and Stuckman, 1979). The microorganism which shows highest lipase activity was designated as BTS-20. The microbial culture was maintained by repeated sub-culturing at 55 °C on a Mineral Based (MB) medium, supplemented with 0.5% (w/v) sucrose and 1.0% (v/v) of cottonseed oil as a sole carbon

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source (pH 7.5). Further, identification and biochemical characterization was done by Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh. It was identified as *Bacillus licheniformis* MTCC 10498. Glycerol stocks were prepared (25%, v/v) and stored at -20°C till further use.

II.(C). Assay of lipase activity and unit of lipase activity

Lipase assays were performed by a colorimetric method. The absorbance of *p*-nitrophenol released was measured at 410_{nm} (Lab India, UV/ Visible spectrophotometer, India). The enzyme activity was defined as µmole(s) of *p*-nitrophenol released per minute by one ml of free enzyme or per gram of immobilized enzyme (weight of matrix included) under standard assay conditions. Specific activity was expressed as µmole(s) of the *p*-nitrophenol released per min per mg of protein.

II.(D). Production of lipase through culture conditions

A loopful of *Bacillus licheniformis* MTCC-10498 culture taken from the MB-agar slant was aseptically transferred into 50 ml (250 ml Erlenmeyer's flask) of MB broth supplemented with sucrose (0.5%, w/v). The seeded broth was incubated at 55°C in a shaking incubator at 150 rpm up to 36 h. All experiments were performed in triplicates unless otherwise stated and mean values were presented. The MB broth 50 ml taken into 250 ml Erlenmeyer flasks in different experiments was inoculated with 10 % (v/v) of 36 h old seed culture. The broth was incubated at 55 °C under shaking (150 rpm). After 72 h of incubation, the lipase activity was assayed in cell-free supernatant obtained after centrifugation at 10,000 x g. The optimized broth containing 0.5 (%; w/v) yeast extract, 0.3% sodium nitrate, and Tween 80 (0.5%, w/v) was calibrated to a final pH of 7.5 to determine the cumulative effect of all the selected components on lipase production by *B. licheniformis* MTCC 10498. The MB broth was autoclaved at 1.1 bar for 20 min. at 121 °C. This broth was inoculated with 10% (v/v) of 36 h old seed culture and incubated under shaking at 55 ºC for 72 h. The inoculated MB broth was harvested at 72 h by centrifugation (10, 000 X g for 20 min. at 4 ºC; Sigma 3K30, Germany). The supernatant was filtered through Whatman filter paper no. 1. This enzyme preparation was termed crude lipase. The lipase produced by *B. Licheniformis* MTCC-10498 in various batches was recorded. The pH was adjusted to 7.5 ± 0.2 and the final volume was made to 1000 ml with sterile distilled water.

II.(E). Lipase production under optimized conditions

The extracellular lipase production by *B. licheniformis* MTCC-10498 in the optimized

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medium broth was studied by inoculating the sterile broth with 10% (v/v) of 36 h old inoculum. The seeded broth was incubated at 55 ± 1 ºC for 72 h under continuous shaking (120 rpm). The lipase production in different batches (n=5) was recorded. The culture broth was further processed to obtain purified lipase. The absorbance of the protein sample was measured at 280_{nm} against the experimental buffer as blank using a spectrophotometer during column chromatography experiments[7]to determine protein concentration in the fractions. A standard quantitative assay for determining the protein content in a solution was used[8]. BSA was used as a reference for protein assay.

II.(F). Purification of *B. licheniformis* MTCC-10498 lipase

The purification of bacterial lipase was performed using techniques of ammonium sulphate salting out, dialysis and hydrophobic interaction chromatography (Octyl-sepharose) respectively. The cell-free supernatant obtained after 36 h of lipase production was used for purification of bacterial lipase. Required amount of ammonium sulphate was added to the supernatant to achieve10 to100% saturation. The contents were thoroughly mixed and kept at 4 ºCover night to achieve maximum precipitation. Thereafter the precipitates were sedimented by centrifugation at 12,000 X g for 30 minutes at 4 ºC. The precipitates were reconstituted in minimal buffer (Tris 0.05 M; pH 8.0) precipitated fractions were analyzed separately for lipase activity and protein content. The precipitates transferred into a dialysis apparatus were extensively dialyzed against Tris buffer (0.05M, pH 8.5) at a regular interval of 2 h so as to completely remove ammonium sulphate. Finally, the lipase was assayed in the dialyzate and was further concentrated using the freeze-drying technique. The concentrated lipase was stored at -20 ºC until further used. A column of pre-swollen Octyl-sepharose (Sigma Chemical Co. MU, Saint Louis, USA) was packed (V_t =25 cm^3) in a sintered glass column. Equilibration was done using 20 ml of start buffer (50 MM) sodium phosphate, 1.0 M ammonium sulphate, pH 7.2) at a flow rate of 1.0 ml/min. The dialyzed lipase was loaded on the column. The elution was performed with 20 ml of elution buffer (50 mM Sodium phosphate, pH 7.2). All fractions (3 ml) were assayed for both lipase activity and protein content. The fraction showing lipase activities under a peak were pooled (14 ml) and quantified. Fold purification as well as yield of lipase was determined at each stage of the purification procedure. Purified lipase was stored at -20 ºC until subsequent use.

II.(G). Determination of molecular mass

Native-PAGE was performed to determine the purity, native molecular mass, and its subunit molecular mass.

The polyacrylamide gel electrophoresis was based on methods.

II.(H). Preparation of gel for Native-PAGE

The electrophoresis system from ATTO Corporation, Japan was used to perform the electrophoresis. To prepare one gel of 1.0 mm thickness for Native-PAGE, 6 ml of separating gel mixture was poured between the two glasses plates sandwiched together with a 1.0 mm spacer. The gel solution was overlaid with n-butanol water and allowed to polymerize for 30 min. The water layer at the top was removed using filter paper and 1 ml of stacking (4%) gel mixture was poured over it. The comb was inserted carefully to avoid air bubbles and kept for 1h for polymerization. The composition used were separating gel (12%) and stacking gel (4%).

II.(I). Native-protein molecular weight marker

The high molecular weight-native protein markers (Bangalore Genei Pvt. Ltd., Bangalore) were used to determination of the native molecular weight of bacterial isolate of *Bacillus licheniformis* MTCC-10498. The molecular weight of markers ranged from 20 kDa to 232kDa.

II.(J). Sample loading and electrophoresis

In the case of native-PAGE, the samples were mixed with the 5x sample loading buffer (without SDS). The prepared samples were loaded onto the gel and electrophoresis at a constant voltage of 100 V in the case of SDS-PAGE until the dye front reached 0.5 cm above the bottom of the gel. The native-PAGE electrophoresis was performed at a constant current of 40 mA.

II. (K). Procedure

After electrophoresis, the gel was carefully transferred to a clean gel staining box containing Coomassie Brilliant Blue R-250 gel stain (50 ml), and was kept over a rocker for agitation for 10-20 min at room temperature. The staining solution was poured off and washed twice with water. The de-staining solution (about 50 ml) was added and gently shaken over the rocker platform for 1 h. Further, changes to the de-staining solution were made, and finally, the gel was left to de-stain overnight.

II.(L). Documentation of gel

The images of gel were recorded in a gel documentation system (Alpha Innotech Corporation, USA). The molecular weight analysis was done using Alpha Digi Doc RT and Alpha Ease FC software.

II.(M). Assay of *in situ* lipase activity in the native- PAGE

In situ lipase activity studies were conducted with the other half portion of the Native-PAGE. After Native-PAGE, the gel was sliced into

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two parts, one of which was subjected to the staining and de-staining procedures mentioned above, and the other part was washed twice with distilled water, and retention of enzymatic activity was analysed by placing a Whatman filter paper No. 1 strip soaked in p-NPP over the gel in close contact. The development of yellow colour on the substrate-soaked filter paper, if any was indicative of the lipolytic activity of the gel-embedded protein. Alternatively, the band reflecting lipase activity was cut with a surgical blade; the gel piece was minced and assayed for associated lipase activity using p-NPP.

III. RESULTS

III.(A). Purification of crude lipase

The cell-free broth when saturated with varying concentrations of ammonium-sulphates resulted in maximal lipase activity in the pellet obtained using 80% (w/v) of ammonium sulphates. The sedimented precipitates were reconstituted in 10 ml of Tris buffer (0.05 M, pH 8.0). This suspension was transferred into a dialysis bag was subjected to extensive dialysis against Tris buffer (0.05 M, pH 8.0). The dialyzate showed lipase activity of 116.57 U/ml (protein content 3.71 mg/ml, and specific activity of 31.38 U/mg) that indicated an approximate 31.06 fold concentration of lipase. The purification of dialyzed lipase on the Octyl-sepharose column resulted in a single peak. The fractions under the single peak correspond to total lipase activity of 588 U (2.0 IU/ml). This hydrophobic–column purified lipase was further evaluated for its homogeneity in on electrophoresis.

III.(B). Electrophoretic characterization of *Bacillus licheniformis* MTCC-10498 lipase: Native–Polyacrylamide gel electrophoresis

A single band 38 KDa was observed in native-PAGE. This band was used to assay lipase activity *in situ.* This protein band was carefully cut and removed. The gel containing the said band was converted to small pieces and was assayed colorimetrically for lipase activity (1.8 U/ml), using p-NPP as substrate (Fig. 1).

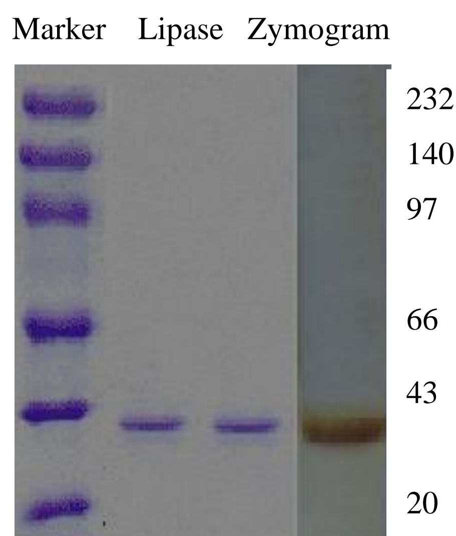

Fig. 1: Native –PAGE with zymogram from *Bacillus licheniformis* MTCC 10498 lipase

It confirmed that a holo-enzyme which contains 2 identical subunits of approximately 19 kDa. The *in situ* substrates (p-NPP) hydrolysis study

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showed that most of the enzymatic activity was retained by the protein in a non-reducing native gel as the cut gel when incubated with 0.05 M Tris buffer (pH 8.0) containing p-nitrophenyl palmitate (previously dissolved in isopropanol) yielded yellow colour.

IV. DISCUSSION

A purified and concentrated lipase preparation was essential to be obtained before the characterization of an enzyme. An impure (crude) lipase preparation could predict undesirable and ambiguous results. In the last few years, emphasis was given to the purification and characterization of bacterial lipases. Previously, different purification procedures were attempted to obtain homogenous lipase preparations[10]. Most of the purification techniques involved fractionation by salting out using ammonium sulphates or the use of an organic solvent. However, ammonium sulphate method was relatively inexpensive, efficient, reliable, and reversible and a general storage technique employed in an enzyme system. The potential advantage of the ammonium sulphate precipitation over all other techniques is the increase in the stabilization of the protein. A 2-3 M ammonium sulphate suspension of protein precipitates remains stable for years, thus it forms a normal packaging method for many enzymes. Also, high salt concentration prevents proteolysis and bacterial activation[11]. Amongst bacteria, to date, a few extracellular lipases have been isolated, characterized, and studied for their catalytic activities in aqueous or their organic media. In general, many of the *Bacillus* lipases have molecular weight ranges from 20-112kDa. A recently reported thermophillic *Bacillus coagulans* BTS-3 isolate possessed an extracellular (31 kDa) alkalophilic lipase[13].

ACKNOWLEDGMENTS

The financial assistance (as UGC RFSMS-SRF, 2008-2012) from the University Grants Commission, New Delhi 110 002, to one of the author Chander Kant Sharma and the funds provided by the Department of Biotechnology, Government of India to the Department of Biotechnology, Himachal Pradesh University, Shimla (India) are thankfully acknowledged.

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